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PCT/9B2005/0005T8



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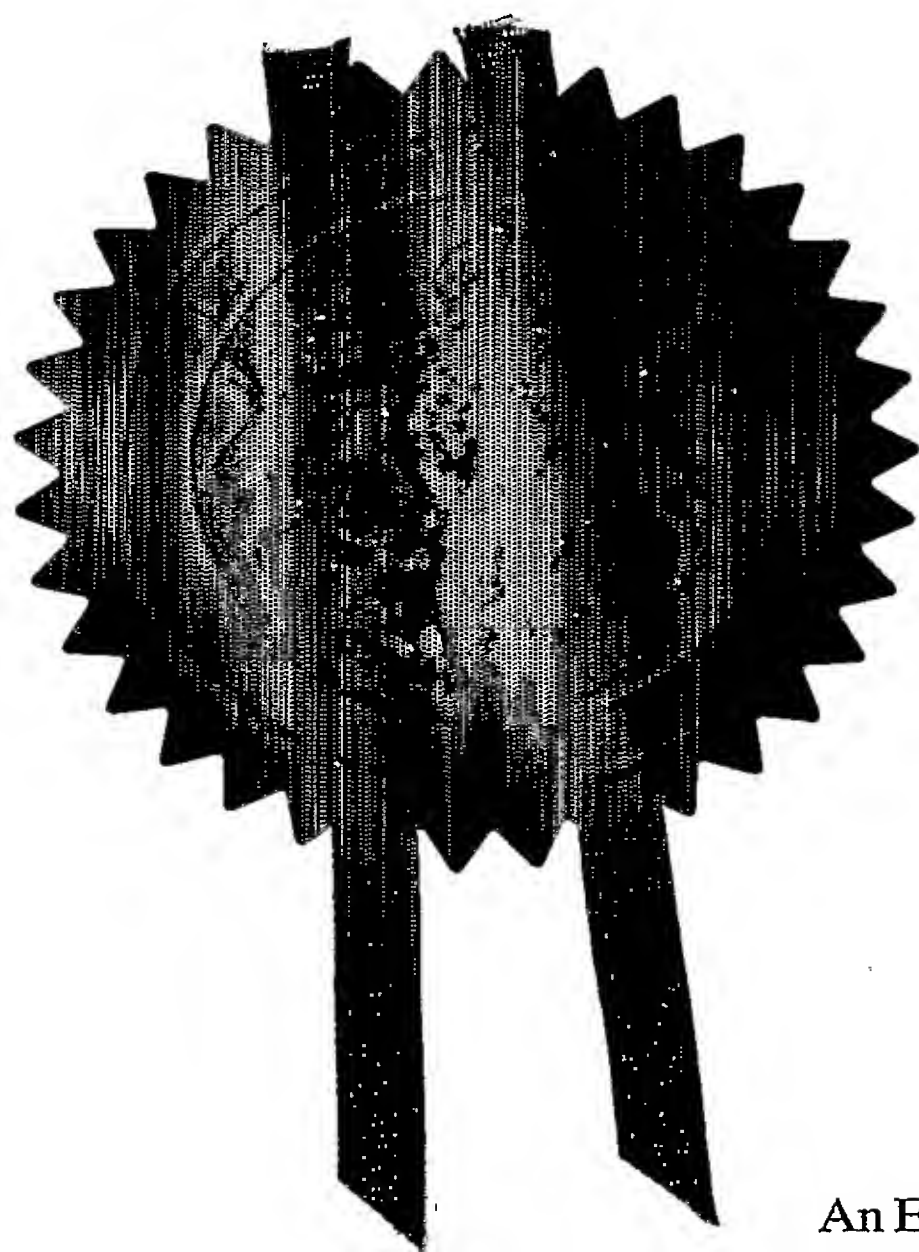
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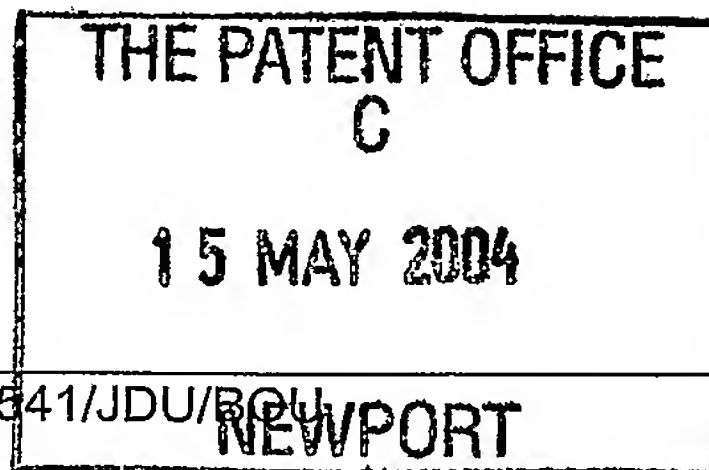
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0410910.4

17MAY04 EBP6436-1 002481

P01/7700 0.00-0410910.4 NONE

3. Full name, address and postcode of the or of each applicant (underline all surnames)

University of Newcastle Upon Tyne,
6 Kensington Terrace
Newcastle Upon Tyne
NE1 7RU

15 MAY 2004

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

6137044009

4. Title of the invention

"Stem Cells"

5. Name of your agent (if you have one)

MURGITROYD & COMPANY

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

SCOTLAND HOUSE
165-169 SCOTLAND STREET
GLASGOW
G5 8PL
UNITED KINGDOM

Patents ADP number (if you know it)

1198013

1198015

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Country

Priority application number
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| Description | 36 |
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Signature(s)



Date 14 MAY 2004

12. Name, daytime telephone number and e-mail address, if any, of person to contact in the United Kingdom

BEVERLEY OUZMAN
beverley.ouzman@murgitroyd.com
+44 (0) 141 307 8400

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1 Stem Cells

2

3 The present invention relates to the culture of
4 primate embryonic stem cells, to the provision of
5 feeder cells of human origin to support embryonic
6 stem cell culture, and to the provision of
7 fibroblast cells for therapeutic use.

8

9 Embryonic stem cells are undifferentiated cells
10 able to proliferate for long periods and which can
11 be induced to differentiate into any type of adult
12 cell.

13

14 Human embryonic stem (hES) cells represent a great
15 potential source of various cell types for
16 therapeutic uses, pharmokinetic screening and
17 functional genomics applications (Odorico et al.,
18 2001, Stem Cells 19:193-204; Schuldiner et al.,
19 2001, Brain Res 913:201-205; Zhang et al., 2002,
20 Nat Biotechnol 19:1129-1133; He et al., 2003, Circ
21 Res 93:32-39).

22

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1 Typically embryonic stem cells are obtained from an
2 embryo at the blastocyst stage (5 to 7 days), by
3 extraction of the inner cell mass (ICM). The ICM
4 is a group of approximately 30 cells located at one
5 end of the internal cavity of the blastocyst.

6 Pluripotent hES cell lines have been obtained from
7 the ICM of Day 5 to 7 blastocysts (Thomson et al.,
8 1998, Science 282:1145-1147; Reubinoff
9 et al., 2000 Nature Biotechnol 18:399-404; Richards
10 et al., 2002, Nature Biotechnol 20:933-936; Hovatta
11 et al., 2003, Hum Reprod 18:1404-1409; Mitalipova
12 et al., 2003, Stem Cells 21:521-526) but to date
13 there have been no reports of obtaining hES cells
14 from older blastocysts due to the difficulty of
15 maintaining the viability of the blastocysts *in*
16 *vitro*.

17
18 Continuous culture of embryonic stem cells in an
19 undifferentiated (pluripotent) state requires the
20 presence of feeder layers such as mouse embryonic
21 fibroblast (MEF) cells (Thomson et al., 1998,
22 Science 282:1145-1147; Reubinoff et al., 2000, Nat
23 Biotechnol 18:399-404), STO cells (Park et al.,
24 2003, Bio Reprod 69:2007-2017), human foreskin
25 fibroblasts (Hovatta et al., 2003, Hum Reprod
26 18:1404-14069) human adult fallopian tubal
27 epithelial cells, human fetal muscle and human
28 fetal skin cells (Richards et al. 2002, Nature
29 Biotechnol 20:933-935), or adult skin fibroblast
30 cell lines (Richards et al. 2003, Stem Cells
31 21:546-556). Alternatively, the culture media can
32 be conditioned by growing the feeder cells in the

1 medium and then harvesting the medium for
2 subsequent stem cell culture (see WO-A-99/20741).
3 Whilst this method is referred to as "feeder-free"
4 culture, nonetheless there is still a reliance on
5 the feeder cells to culture isolated ICMs and to
6 condition the media and hence there is potential
7 for pathogen transmission.

8
9 Unfortunately the use of feeder cells for the
10 culture of hES cells limits their medical
11 application for several reasons: xenogeneic and
12 allogeneic feeder cells bear the risk of
13 transmitting pathogens and other unidentified risk
14 factors (Richards et al., 2002, Nat Biotechnol
15 20:933-936; Hovatta et al., 2003, Hum Reprod
16 18:1404-1409). Also, not all human feeder cells
17 and cell-free matrices support the culture of hES
18 cells equally well (Richards et al., 2002, Nat
19 Biotechnol 20:933-936; Richards et al., 2003, Stem
20 Cells 21: 546-556), and the availability of human
21 cells from aborted fetuses or Fallopian tubes is
22 relatively low. Additionally there are ethical
23 concerns regarding the derivation of feeder cells
24 from aborted human fetuses.

25
26 For example, WO-A-03/78611 describes a method of
27 culturing human fibroblasts delivered from aborted
28 human fetuses, typically of 4 to 6 week gestation.
29 The fibroblasts are cultured from the rib region of
30 the embryo and are described as being suitable to
31 support human embryonic stem cell culture. However

1 this method relies upon the donation of aborted
2 fetuses to maintain a supply of fibroblasts.
3 US-A-2002/0072117 and US 6,642,048 describe the
4 production of a human embryonic stem cell line by
5 culturing the ICM of blastocysts and subsequently
6 inducing the embryonic stem cells to form embryoid
7 bodies and to differentiate into a mixed
8 differentiated cell populations. Cells having a
9 morphology typical of fibroblasts were selected for
10 use as feeder layers or to condition cell culture
11 media for feeder-free culture. However no markers
12 typical of fibroblasts were noted as being present
13 on these cells.

14
15 There remains a need to culture primate embryonic
16 stem (pES) cells, especially hES cells intended for
17 therapeutic use, using only feeder cells of the
18 same species or media conditioned by such feeder
19 cells, to reduce the risk of cross-species pathogen
20 transmission. Additionally, as mentioned above,
21 the use of aborted fetuses as a source of human
22 feeder cells is recognised to be of ethical concern
23 and an alternative source of suitable feeder cells
24 is required.

25
26 The present invention provides a novel human
27 embryonic stem (hES) cell line. The novel cell
28 line is termed hES-NCL1.

29
30 The hES cell line described above was isolated
31 using novel methodology, which forms a further
32 aspect of this invention, and was noted to

1 spontaneously differentiate into fibroblast-like
2 cells in the absence of any trigger and without the
3 formation of embryoid bodies. The fibroblast-like
4 cells so formed expressed the specific fibroblast
5 marker AFSP (anti-fibroblast cell surface specific
6 protein, from Sigma). A photomicrograph of the
7 stained fibroblast-like cells is shown at Figures
8 2B, C, D. The stem cell derived fibroblast-like
9 cells, their formation and their use in culture (as
10 feeder cells or to condition the culture media) of
11 animal embryos (including non-human embryos such as
12 non-human primate embryos as well as human embryos)
13 or embryonic or non-embryonic stem cells (which
14 embryonic or non-embryonic stem cells may be of
15 human or non-human origin), and in therapy forms a
16 further aspect of the present invention and is
17 discussed further below.

18
19 In one aspect, the present invention provides a
20 method of culturing a blastocyst, said method
21 comprising exposing said blastocyst to Buffalo rat
22 liver cells or media conditioned thereby for at
23 least 12 hours.

24
25 The Buffalo rat liver cells may conveniently be
26 present in the cell culture media or, more
27 preferably, will be used to condition that media.

28
29 The blastocyst may be exposed to the Buffalo rat
30 liver cells or media conditioned thereby for a
31 minimum period of 24 hours, 36 hours, 48 hours, 60

1 hours or 72 hours. We have found that an exposure
2 period of approximately 2 days is sufficient.
3 Where the blastocyst is to be used to generate
4 pluripotent embryonic stem cells, it is desirably
5 exposed to the Buffalo rat liver cells or media
6 conditioned thereby in the period immediately prior
7 to the extraction of cells of the ICM. Benefits
8 may also be obtained from exposing the blastocyst
9 to Buffalo rat liver cells or media conditioned
10 thereby where it is intended for preimplantation as
11 part of IVF treatment.

12

13 In more detail, one protocol for culturing a
14 blastocyst according to the present invention
15 comprises:

- 16 i) culturing said blastocyst from fertilisation
17 in G1 media;
18 ii) transferring said blastocyst of step i) to
19 G2.3 media and maintaining said blastocyst in
20 the G2.3 media; and
21 iii) transferring said blastocyst of step ii) to
22 cell culture media conditioned by Buffalo rat
23 liver cells.

24

25 The G1 and G2.3 media referred to above can be
26 obtained from Vitrolife Sweden AB, Kungsbacka,
27 Sweden.

28

29 G-1TM is a media designed to support the
30 development of embryos to the 8-cell stage, ie.
31 from pro-cleavage to day 2 or 3. The media
32 contains carbohydrates, amino acids and chelators,

1 as well as Hyaluronan and is bicarbonate buffered.

2 In more detail, the G-1TM media contains:

| | |
|-----------------------|-----------------------------|
| 3 Alanine | Penicillin G |
| 4 Alanyl-glutamine | Potassium chloride |
| 5 Asparagine | Proline |
| 6 Aspartate | Serine |
| 7 Calcium chloride | Sodium bicarbonate |
| 8 EDTA | Sodium chloride |
| 9 Glucose | Sodium dihydrogen phosphate |
| 10 Glutamate | Sodium lactate |
| 11 Glycine | Sodium pyruvate |
| 12 Hyaluronan | Taurine |
| 13 Magnesium sulphate | Water for injection (WFI) |

14

15 G-2TM is a cell culture media to support the
 16 development of embryos from around the 8-cell stage
 17 to the blastocyst stage. The media contains
 18 carbohydrates, amino acids and vitamins, as well as
 19 Hyaluronan, and is bicarbonate buffered. In more
 20 detail the G-2TM version 3 (ie. G2.3) media
 21 contains:

22

| | |
|-------------------------|--------------------|
| 23 Alanine | Penicillin G |
| 24 Alanyl-glutamine | Phenylalanine |
| 25 Arginine | Potassium chloride |
| 26 Asparagine | Proline |
| 27 Aspartate | Pyridoxine |
| 28 Calcium chloride | Riboflavin |
| 29 Calcium pantothenate | Serine |
| 30 Cystine | Sodium bicarbonate |
| 31 Glucose | Sodium chloride |

| | | |
|----|--------------------------------------------------------------|-----------------------------|
| 1 | Glutamate | Sodium dihydrogen phosphate |
| 2 | Glycine | Sodium lactate |
| 3 | Histidine | Sodium pyruvate |
| 4 | Hyaluronan | Thiamine |
| 5 | Isoleucine | Threonine |
| 6 | Leucine | Tryptophan |
| 7 | Lysine | Tyrosine |
| 8 | Magnesium sulphate | Valine |
| 9 | Methionine | Water for injection (WFI) |
| 10 | | |
| 11 | The duration of step i) above may typically be from | |
| 12 | Day 0 (at fertilisation) to Day 3. | |
| 13 | | |
| 14 | The duration of step ii) above may typically be for | |
| 15 | 2 or 3 days, that is from Day 3 to Day 5 or 6. | |
| 16 | | |
| 17 | The duration of step iii) above is for a minimum | |
| 18 | period of 24 hours as described above, but may | |
| 19 | typically be for 1 to 3 days. | |
| 20 | | |
| 21 | In step iii) a preferred cell culture media | |
| 22 | consists of Dulbecco's modified Eagle's medium | |
| 23 | (DMEM, Invitrogen, Paisley, Scotland), optionally | |
| 24 | supplemented with 15% (v/v) Glasgow medium, and | |
| 25 | conditioned by Buffalo rat liver cells (see | |
| 26 | Stojkovic et al., 1995, Biol Reprod 53:1500-1507). | |
| 27 | Typically conditioning by the Buffalo rat liver | |
| 28 | cells comprises culturing 75000 buffalo rat liver | |
| 29 | cells/cm ² in Glasgow medium for 24-36 hours. The | |
| 30 | media is then recovered and frozen at -20°C until | |
| 31 | required. | |

1 Using a blastocyst cultured as described above, the
2 ICM can be extracted using routine techniques as
3 late as Day 8, typically by immunosurgery (see
4 Reubinoﬀ et al., 2001, Hum Reprod 10:2187-2194).
5 Blastocysts were cultured for 30 minutes in whole
6 human antiserum (Sigma) diluted 1:5 in DMEM+FCS
7 medium (i.e. 80% Dulbecco's modified Eagle's medium
8 with 10-20% (v/v) fetal calf serum). Furthermore,
9 the blastocysts were washed three times and
10 cultured for another period of approximately 20
11 minutes in guinea pig complement (1:5). The
12 isolated ICMs were used for embryonic stem cell
13 culture but could alternatively be implanted into a
14 receptive female as part of an IVF treatment.

15
16 For human blastocysts, the blastocyst will have
17 been donated, with informed consent, as being
18 superfluous to IVF treatment. For other (ie. non-
19 human) primates, the ovulation cycle can be
20 controlled by intramuscular injection of
21 prostaglandin or a prostaglandin analogue, and the
22 embryos harvested by a non-surgical uterine flush
23 procedure (see Thompson et al., 1994, J Med
24 Primatol 23:333-336) at day 8 following ovulation.

25
26 If the blastocyst is unhatched, the zona pellucida
27 is removed by brief exposure to pronase. This step
28 is not required for hatched embryos. The
29 blastocyst is exposed to antiserum for 30 minutes.
30 The blastocyst is then washed three times in DMEM,
31 and exposed to a 1:5 dilution of Guinea pig
32 complement (Gibco) for 20 minutes. After two

1 further washes in DMEM, lysed trophectoderm cells
2 are removed from the ICM by pipette and the ICM
3 plated out on a suitable feeder layer. Embryonic
4 stem cell lines are identified from the cultured
5 ICM cells.

6
7 As mentioned above, the novel methodology enables
8 the blastocyst to be cultured at a relatively late
9 stage, day 8. At day 8 the number of cells
10 obtainable from the ICM is considerably increased,
11 but surprisingly these cells retain their
12 pluripotent ability.

13
14 The present invention therefore provides a method
15 of producing an embryonic stem cell line, said
16 method comprising:

- 17 i) culturing a blastocyst as described above; and
18 ii) extracting cells of the ICM from said
19 blastocyst and culturing the cells to produce
20 an embryonic stem cell line therefrom.

21
22 The reference to culturing the cells of the ICM
23 extracted from the blastocyst in step ii) above
24 includes the published protocols available and is
25 not especially dependent upon any particular
26 culture conditions.

27
28 The method of producing stem cells according to the
29 present invention provides a generic and efficient
30 method for the production of primate embryonic stem
31 (pES) cell lines. The pES cell lines may be human
32 embryonic stem (hES) cell lines. Alternatively the

1 pES cells may be of non-human origin. The stem
2 cell lines so produced are preferably of clinical
3 and/or GMP grade.
4

5 One suitable medium for the isolation of embryonic
6 stem cells consists of 80% Dulbecco's modified
7 Eagle's medium (DMEM; obtainable from Invitrogen or
8 Gibco) with 10-20% (v/v) fetal calf serum (FCS,
9 Hyclone, Logan, UT). Optionally the medium may
10 also include one or more of 0.1 mM β -
11 mercaptoethanol (Sigma), up to 1% (v/v) non-
12 essential amino acid stock (Gibco), 1% (v/v)
13 antibiotic, such as penicillin-streptomycin
14 (Invitrogen), and/or 4ng/ml bFGF (Invitrogen). To
15 date details of several specific media suitable for
16 embryonic stem cell culture have been published in
17 the literature - see for example Thomson et al.,
18 1998, Science 282:1145-1147; Xu et al., 2001,
19 Nature Biotechnol 19:971-974; Richards et al.,
20 2002, Nature Biotechnol 20:933-936; and Richards et
21 al., 2003, Stem Cells 21:546-556.
22

23 Feeder cells which may be used for stem cell
24 culture include mouse embryonic stem cells (MEF),
25 STO cells, foetal muscle, skin and foreskin cells,
26 adult Fallopian tube epithelial cells (Richards et
27 al., 2002, Nat Biotechnol 20:933-936; Amit et al.,
28 2003, Biol Reprod 68:2150-2156; Hovatta et al.,
29 2003, Hum Reprod 18:1404-1409; Park et al., 2003,
30 Biol Reprod 69, 2007-2014; Richards et al., 2003,
31 Stem Cells 21:546-556), adult bone marrow cells
32 (Cheng et al., 2003, Stem Cells 21:131-142), or on

1 coated dishes with animal based ingredients with
2 the addition of MEF cell conditioned media (Xu et
3 al., 2001, Nature Biotechnol 19:971-974).

4
5 The method of culturing a blastocyst and the method
6 of producing embryonic stem cell lines as described
7 above are both suitable for use with blastocysts of
8 primate origin, including blastocysts of human or
9 non-human origin.

10
11 The human embryonic stem cells of the present
12 invention are characterised by at least one of the
13 following;

- 14 i) presence of the cell surface markers TRA-1-60,
15 GTCM2, and SSEA-4;
16 ii) expression of *Oct-4*;
17 iii) expression of *NANOG*;
18 iv) expression of *REX-1*; and/or
19 v) expression of *TERT*.

20
21 In one embodiment at least 2 or more of the
22 characteristics listed above are present,
23 preferably 3 or more of the characteristics are
24 present, especially 4 or more, more preferably all
25 of the above characteristics are present in the
26 stem cells.

27
28 The antigen SSEA-4 is a glycolipid cell marker.
29 Specific antibodies to identify this marker are
30 available from the Development Studies Hybridoma
31 Bank, DSHB, Iowa City, IA.

32

1 The cell surface marker TRA-1-60 is recognised by
2 antibodies produced by hybridomas developed by
3 Peter Andrews of the University of Sheffield (see
4 Andrews et al., "Cell lines from human germ cell
5 tumours" pages 207-246 in Teratocarcinomas and
6 Embryonic Stem Cells: A Practical Approach, Ed.
7 Robertson, Oxford, 1987). TRA1-60 is also
8 commercially available (Chemicon). Both GTCM2 and
9 TG343 are described in Cooper et al., 2002, J.
10 Anat. 200(Pt 3):259-65.

11
12 The embryonic stem cell line produced according to
13 the method of the present invention as described
14 above (and specifically the stem cell line hES-
15 NCL1) can be used for screening and/or to produce
16 differentiated cells of specific cell types for
17 therapeutic purposes (e.g. for implantation to
18 replace damage or missing tissue). The stem cell
19 lines (e.g. hES-NCL1) can be used to screen agents
20 (e.g. chemical compounds or compositions) for
21 toxicity and/or for therapeutic efficacy (i.e.
22 pharmacological activity).

23
24 In a further aspect, the present invention provides
25 a method of screening an agent for toxicity and/or
26 for therapeutic efficacy, said method comprising:

- 27 a) exposing an embryonic stem cell line
28 obtained according to the method described
29 (e.g. hES-NCL1) to said agent;
30 b) monitoring any alteration in viability
31 and/or metabolism of said stem cells; and

1 c) determining any toxic or therapeutic effect
2 of said agent.

3
4 Additionally, the method of producing a stem cell
5 line according to the present invention as
6 described above, and the stem cell lines produced
7 thereby (e.g. hES-NCL1) may be used in the creation
8 of an embryonic stem cell bank for use in screening
9 and/or to produce differentiated cells of specific
10 cell types for therapeutic purposes. The stem cell
11 bank, which forms a further aspect of the present
12 invention, will consist of a multiplicity of
13 genetically distinct stem cell lines. The stem
14 cell lines forming the stem cell bank will usually
15 be of primate embryonic stem cells such as human
16 embryonic stem cells or non-human embryonic stem
17 cells. The embryonic stem cell bank can be used to
18 screen agents (e.g. chemical compounds or
19 compositions) for toxicity and/or for therapeutic
20 efficacy (i.e. pharmacological activity).

21
22 Thus, in a yet further aspect, the present
23 invention provides a method of screening an agent
24 for toxicity and/or for therapeutic efficacy, said
25 method comprising:

26 a) exposing an embryonic stem cell bank
27 comprising a multiplicity of embryonic stem
28 cell lines obtained according to the method of
29 the present invention to said agent;
30 b) monitoring any alteration in viability and/or
31 metabolism of said stem cells; and

1 c) determining any toxic or therapeutic effect of
2 said agent.
3

4 As briefly mentioned above, it was noted that the
5 embryonic stem cell line established from a
6 blastocyst cultured as described above according to
7 the present invention spontaneously differentiated
8 into fibroblast-like cells without formation of
9 embryoid bodies. Such spontaneous differentiation
10 into a single cell type was unexpected. These
11 fibroblast-like cells then acted as a feeder layer
12 for the remaining undifferentiated embryonic stem
13 cells of the culture. The stem cell derived
14 fibroblast-like cells and the embryonic stem cells
15 supported thereby were autogeneic.
16

17 The spontaneous differentiation of hES cells in a
18 feeder-free culture into a mixture of cell types,
19 including fibroblast-like cells, has already been
20 described (see Park et al., 2003, Biol Reprod
21 69:2007-2014) but in that study the differentiation
22 was observed in the centre of the hES cell
23 colonies. This differs to the present invention
24 where differentiation occurs at the periphery of
25 the colony. Moreover in the present invention only
26 fibroblast-like cells were observed and no other
27 cell types were noted to be present.
28

29 The present invention therefore provides a method
30 of producing fibroblast-like cells, said method
31 comprising:

32 i) culturing a blastocyst as described above;

- 1 ii) extracting cells of the ICM from said
- 2 blastocyst and culturing the cells to produce
- 3 an embryonic stem cell line therefrom; and
- 4 iii) allowing cells of said embryonic stem cell
- 5 line to differentiate into stem cell derived
- 6 fibroblast-like cells.

7

8 The stem cell derived fibroblast-like cells are

9 produced without requiring a specific stimulant,

10 e.g. growth factor or change in physical growth

11 conditions (e.g. allowing the cells to become

12 crowded).

13

14 One suitable method for obtaining differentiation

15 of the stem cells into fibroblast-like cells was

16 simply to transfer the stem cells to cell culture

17 media in the absence of feeder cells or feeder cell

18 conditioning. The stem cells responded by

19 differentiation of a proportion of the stem cells

20 which then acted as feeder cells for the non-

21 differentiated remaining stem cells. Thus

22 obtaining differentiation into fibroblast-like

23 cells was possible using an extremely easy one-step

24 process, avoiding the need for time-consuming

25 procedures and allowing the differentiation to be

26 fully controlled under *in vitro* conditions.

27

28 The stem cell derived fibroblast-like cells are

29 characterised by a morphology typical of the cell

30 type, ie. long flat cells with an elongated,

31 condensed nucleus. The cytoplasmic processes

1 therein resemble those found in fibroblasts of
2 connective tissue.

3
4 The fibroblast-like cells of the present invention
5 are positive for the cell surface marker AFSP. In
6 addition, the identity of hES cells-derived
7 fibroblasts was confirmed by karyotyping and DNA
8 analysis of both stem cells and hES cells-derived
9 fibroblasts. This confirmed that hES cells-derived
10 fibroblasts are autogeneic i.e. of the same origin
11 as the stem cells.

12
13 The fibroblast-like cells according to the present
14 invention could be easily immortalised using known
15 techniques to provide a long term source of the
16 cells.

17
18 The present invention also provides a novel human
19 embryonic stem cell derived fibroblast-like cell
20 line. The novel fibroblast-like cell line, termed
21 hESCdF-NCL, has been deposited at the European
22 Collection of Cell Cultures on 19 January 2004
23 under Accession No 04010601.

24
25 The fibroblast-like cells and media conditioned by
26 the fibroblast-like cells of the present invention
27 are suitable to support the growth of embryos. The
28 fibroblast-like cells and media conditioned by the
29 fibroblast-like cells of the present invention are
30 alternatively suitable to support the growth of
31 stem cells, especially non-human primate embryonic
32 stem cells or human embryonic stem cells. Other

1 types of stem cells needing the use of feeder cells
2 to survive are also included and particular mention
3 may be made of unipotential and pluripotential stem
4 cells such as adult stem cells, haemopoietic stem
5 cells, mesenchymal stem cells, osteogenic stem
6 cells, chondrogenic stem cells, neuronal stem
7 cells, gonadal stem cells, epidermal stem cells and
8 somatic/progenitor stem cells. Where the
9 fibroblast-like cells of the present invention are
10 used to support human stem cells, the fibroblast-
11 like cells are desirably autogeneic thereto but
12 xenogeneic feeder cells may be used following
13 screening to ensure that they are pathogen-free.

14

15 In a further aspect, the present invention provides
16 a self-feeder system for the growth of
17 undifferentiated stem cells, said system comprising

18 i) culturing a blastocyst as described above;

19

20 ii) extracting cells of the ICM from said
21 blastocyst and culturing the cells to produce
22 an embryonic stem cell line therefrom; and

23

24 iii) and allowing some of the cells of said
25 embryonic stem cell line to differentiate
26 into stem cell derived fibroblast-like cells
27 whilst the remainder of the cells of said
28 embryonic stem cell line remain in an
29 undifferentiated, pluripotent state, whereby
30 said stem cell derived fibroblast-like cells
31 act as autogeneic feeder cells for said stem
32 cells.

1
2 The fibroblast-like cells may be used directly as
3 feeder cells to support stem cell culture (eg are
4 grown as a confluent surface in contact with the
5 stem cells) or may be used to condition media for
6 use in stem cell culture. Generally, where the
7 media is to be conditioned, the fibroblast-like
8 cells are grown in the media for a predetermined
9 period of typically 24 hours, although periods of
10 up to a maximum of 9 days may be used, before the
11 media is removed and transferred to the stem cells.
12
13 There are several advantages for using hES cells
14 derived fibroblasts as feeder cells: i) feeder
15 derived from hES cells offers more secure
16 autogeneic/genotypically homogenous system for
17 prolonged growth of undifferentiated hES cells, ii)
18 feeders differentiated from first clinical-grade
19 hES cell line could be used worldwide as initial
20 monolayer for growth of isolated ICMs to eliminate
21 transfer of pathogens, iii) the long proliferation
22 time of already derived hES cell lines allows
23 screening for viral contamination, iv) medium
24 conditioned by hESdF can be used for feeder-free
25 growth of hES cells thus avoiding potential viral
26 transfer from the MEF conditioned media used to
27 date, v) due to the low bioburden, embryonic
28 tissues perform better support *in vitro* than adult
29 tissues (see Richards et al., 2003, Stem Cells
30 21:546-556), vi) derivation and culture of hESdF is
31 fully controlled and not time consuming, vii)
32 derived feeder cells could be easily immortalized

1 to provide a long-term source of this tissue, viii)
2 *in vitro* studies on cell-to-cell contacts and
3 identification of isolated soluble factors could
4 significantly improve cell-culture, cell-
5 transplantation and tissueengineering avoiding at
6 the same time expensive tissue-biopsy and
7 unnecessary sacrifice of animals.

8
9 Accordingly, the present invention further provides
10 a method of culturing a primate embryonic stem cell
11 line, such as a human embryonic stem cell line, to
12 maintain the viability of eggs prior to or during
13 fertilisation and/or to culture blastocysts or
14 embryos intended for implantation into a receptive
15 female to establish a pregnancy (i.e. as part of an
16 IVF procedure). The method comprises providing
17 fibroblast-like cells obtained according to the
18 present invention as feeder cells or to condition
19 the cell culture media. Advantageously the
20 fibroblast-like cells selected will be obtained
21 from an embryonic stem cell line of the same origin
22 or species, and will be previously screened to
23 ensure pathogen-free status. This approach enables
24 the complete elimination of animal ingredients for
25 the culture of undifferentiated hES cells and
26 avoids the potential of viral transfer which may
27 occur when MEF conditioned media or conditioned
28 media from other feeders is used for stem cell
29 culture.

30
31 We have found that the use of the fibroblast-like
32 cells obtained according to the present invention

1 (e.g. hESCdF-NCL) as feeder cells or to condition
2 the culture media enables the undifferentiated
3 culture of the embryonic stem cells. It is
4 anticipated that a similar ability will be obtained
5 using other stem cell types. This is highly
6 significant for the long term maintenance of such
7 cell lines and also has the advantage that the
8 extended culture period possible for the
9 undifferentiated stem cell line enables the cell
10 line to be screened for any potential pathogen
11 (e.g. viral contamination).
12

13 Alternatively, the fibroblast-like cells can be
14 used for therapy, for example to assist
15 regeneration of wounds requiring fibroblast
16 presence.
17

18 The presence of fibroblast cells, without
19 contamination of other cell types is of particular
20 advantage in therapy. One example of the use of
21 the fibroblasts according to the present invention
22 is the generation of skin grafts for use in
23 treating wounds (for example burns) or in cosmetic
24 or regenerative surgery.
25

26 The present invention will now be further described
27 with reference to the following examples and
28 figures, in which:
29

30 **Figure 1.** Morphology of human blastocysts and hES
31 cells. Day 6 blastocysts (A) and hatched Day 8
32 blastocysts (B). Note the presence of very well

1 organised inner cell mass in Day 8 blastocyst
2 recovered after three-step *in vitro* culture. Inner
3 cell mass cells (C) grown on irradiated MEF 4 days
4 after immunosurgery. Primary hES cells colony (D)
5 grown on inactivated MEF cells. Same colony at high
6 magnification (E). Bars: 50 μm (A-D); 100 μm (E).

7
8 **Figure 2.** Morphology and characterisation of hES
9 cells-derived fibroblasts. Undifferentiated hES
10 cells (A). Peripheric differentiation of hES cells
11 into fibroblast-like cells in feeder-free
12 conditions (B). Phase (C) and fluorescence (D)
13 microscopy of hES cells-derived fibroblasts using
14 AFSP antibody. Normal 46 + XX karyotypes of hES
15 cells (E) and hES cells-derived fibroblasts (F).
16 Microsatellite analysis of hES cells (G) and hES
17 cells-derived fibroblasts (H). Bars: 50 μm (A, C,
18 D), 100 μm (B).

19
20 **Figure 3.** Morphology of frozen/thawed hES-NCL1
21 colony cultured on frozen/thawed hES cell-derived
22 fibroblasts. Bar: 50 μm .

23
24 **Figure 4.** Morphology and characterisation of hES-
25 NCL1 cells grown on γ -irradiated hESdF monolayer
26 (A-F) or feeder-free (G, H). (A) Five days old
27 vitrified hES-NCL1 colony cultured on frozen/thawed
28 hESdF (passage 8). (B) Higher magnification of the
29 same hES colony. Note typical morphology of hES
30 cells i.e. small cells with prominent nucleoli. hES
31 cells grown on hESdF stained with antibody

1 recognising the TRA1-60 (D) and SSEA-4 (F)
 2 epitopes. HES cells grown on Matrigel (G) with
 3 addition of hESdF conditioned medium stained with
 4 antibody recognising the GTCM2 epitope (H). Bars:
 5 200 μ m (A, E-H); 50 μ m (B); 100 μ m (C, D).

6
 7 **Figure 5.** Characterisation and karyotyping of hES-
 8 NCL1 cells grown on hESdF monolayer. RT-PCR
 9 analysis of undifferentiated hES cells grown on
 10 inactivated hESdF cells (A). PCR products obtained
 11 using primers specific for *OCT-4*, *NANOG*, *FOXD3*,
 12 *TERT*, *REX1* and *GAPDH*. HES cells (passage 31) grown
 13 on hESdF (passage 11) show normal female karyotype
 14 (46, XX) (B).

15
 16
 17 **Figure 6.** Histological analysis of teratomas formed
 18 from grafted colonies of hES cells grown on
 19 inactivated hESdF in testis (A-C) and kidney (D-F)
 20 of SCID mice. (A) neural epithelium (ne); (B)
 21 aggregation of glandular cells with characteristic
 22 appearance of secretory acini (sa); (C) cartilage
 23 (cart); (D) wall of respiratory passage showing
 24 epithelium (ep), submucosa (sm), submucosal glands
 25 (sg). Epithelium contains occasional ciliated cells
 26 and numerous goblet cells secreting mucin (m); (E)
 27 Two types of epithelia: respiratory (top),
 28 keratinised skin (bottom). Submucosal glands (sg)
 29 located beneath pseudostratified ciliated (in
 30 parts) epithelium (ep). Structures of the skin
 31 include epidermis (ed), dermis (dm) and cornified
 32 layer (c). Note that the stratum granulosum (arrow)

is characterised by intracellular granules which contribute to the process of keratinisation. Occasional mitotic indices (m) are seen in the basal layer; (F) High magnification image of skin, showing greater detail of dermis (dm), epidermis (ed) and cornified layer (c). Again the stratum granulosum is visible (arrow). Scale bars: (A, B, C) 100 μm ; (D, E) 25 μm ; (F) 17.5 μm .

Figure 7. Flow cytometry analysis of hESdF (left panel) and human foreskin fibroblasts (HFF, right panel) for the presence of CD31, CD44, CD71, CD90 and CD106. The bold (red) line represents the staining with the isotype control and the grey (green) line staining with specific antibodies.

Figure 8. Spontaneous differentiation of hES-NCL1 cells grown on hESdF and then in feeder-free conditions. hES-NCL1 differentiate into neuronal (A) and smooth muscle (B) cells demonstrating differentiation into cells of ectoderm and mesoderm, respectively. Green: cells stained with nestin antibody (A) and smooth muscle actin antibody (B). Red: cell-nuclei stained with propidium iodide. (A) shows small areas of red and green staining dispersed across the cells in a check-like pattern. (B) shows all cells stained green. Scale bars: 100 μm (A) and 50 μm (B).

1 **Examples**

2

3 **Material and Methods**

4

5 **Culture of embryos.** Two day old human embryos,
6 produced by *in vitro* fertilization (IVF) for
7 clinical purposes, were donated by individuals
8 after informed consent and after Human
9 Fertilisation and Embryology Authority (HFEA, UK)
10 approval. Until Day 3 (IVF = Day 0), 11 embryos
11 were cultured in G1 medium and transferred to G2.3
12 medium (both G1 & G2.3 from Vitrolife, Kungsbacka,
13 Sweden) until day 6. Day 6 recovered blastocysts
14 were cultured in Dulbecco's modified Eagle's medium
15 (DMEM, Invitrogen, Paisley, Scotland) supplemented
16 with 15% (v/v) Glasgow medium conditioned by
17 Buffalo rat liver cells which has been used
18 successfully for the long-term culture of bovine
19 embryos, termed G-BRLC media (Stojkovic et al.,
20 1995, Biol Reprod 53:1500-1507). On Day 8 ICMs
21 were isolated by immunosurgery as previously
22 described (Reubinoﬀ et al., 2001, Hum Reprod
23 10:2187-2194).

24

25 **Cell-number analysis.** We investigated whether our
26 three-step embryo culture supported development of
27 Day 8 blastocysts and whether these blastocysts
28 posses more ICM cells than Day 6 blastocysts.
29 Eleven isolated ICMs from Day 6 blastocysts (5
30 blastocysts and 6 expanded blastocysts) and 13 ICMs
31 from Day 8 blastocysts (7 expanded and 6 hatching
32 or hatched blastocysts) were analysed using 1.5

1 μg/ml 4'-6-diamidino-2-phenylindole (DAPI, Sigma,
2 St. Louis, MO) labelling as previously described
3 (Spanos et al., 2000, Biol Reprod 63:1413-1420).
4

5 **Derivation of hES cells.** Initially, isolated ICMS
6 were cultured on γ-irradiated MEFs monolayer
7 (75.000 cell/cm²) and DMEM supplemented with 10%
8 (v/v) Hyclone defined fetal calf serum (FCS,
9 Hyclone, Logan, UT) for 10 days. After 17 days, the
10 hES cell colony was mechanically dispersed into
11 several small clumps which were cultured on a fresh
12 MEF layer with ES medium containing Knockout-DMEM
13 (Invitrogen), 100 μM β-mercaptoethanol (Sigma), 1
14 mM L-glutamine (Invitrogen), 100 mM non-essential
15 amino acids, 10% serum replacement (SR,
16 Invitrogen), 1% penicillin-streptomycin
17 (Invitrogen) and 4 ng/ml bFGF (Invitrogen). ES
18 medium was changed daily. Human embryonic stem
19 cells were passaged by incubation in 1 mg/ml
20 collagenase IV (Invitrogen) for 5-8 minutes at 37°C
21 or mechanically dissociated and then removed to
22 freshly prepared MEF or hES cells-derived feeders.
23

24 **Recovery of hES cell-derived fibroblasts.** Once a
25 stable stem cell line was established, hES cells
26 were transferred into feeder-free T-25 flasks
27 (Iwaki, Asahi, Japan), using DMEM supplemented with
28 10% FCS at 37°C in a 5% CO₂ atmosphere. After one
29 week the stem cell derived fibroblast-like cells
30 were transferred into T-75 flasks (Iwaki) and
31 cultured for a further 3 days to produce a

1 confluent primary monolayer of hES cells-derived
2 fibroblasts.

3
4 **Immunocytochemical analysis of hES cells and hES**
5 **cells-derived fibroblasts.** Live staining was
6 performed by adding primary antibodies (TRA1-60 and
7 TRA1-81, a kind gift from Prof. P. Andrews
8 (University of Sheffield, UK) (but also available
9 commercially from Chemicon); SSEA-4, SSEA-4 (MC-
10 813-70) from Developmental Studies Hybridoma Bank,
11 DSHB, Iowa City, IA; GCTM-2 and TG343, both a kind
12 gift from Dr. M. Pera (Monash Institute of
13 Reproduction and Development, Clayton, Australia);
14 anti-fibroblast surface protein, AFSP from Sigma)
15 to hES cells and hES cells-derived fibroblasts for
16 20 minutes at 37°C. The primary antibodies were
17 used at the following dilutions: TRA-1-60 - 1:10;
18 TRA1-81 - 1:10; SSEA-3 - 1:4; SSEA-4 - 1:5
19 (Henderson et al., 2002, Stem Cells 20:239-337);
20 GCTM-2 - 1:2; AFSP - 1:50 (Ronnov-Jessen, 1992,
21 Histochem Cytochem 40:475-486). TG343 at 1:2
22 (Cooper et al., 2002, J Anat 200:259-265) was used
23 to label cells grown on MEF feeder cells. The
24 samples were gently washed three times with ES
25 medium before being incubated with the 1:100
26 secondary antibodies (anti mouse IgG and anti mouse
27 IgM, both Sigma) conjugated to fluorescein
28 isothiocyanate (FITC) at 37°C for 20 minutes. The
29 samples were again washed three times with ES
30 medium and subjected to fluorescence microscopy.
31 For the Oct4 immunostaining hES cells were fixed in
32 3.7% formaldehyde BDH, Coventry, UK for 20 minutes

1 at room temperature followed by incubation in 3%
2 hydrogen peroxide for 10 minutes. The hES cells
3 were permeabilised with 0.2 % Triton x100 (Sigma)
4 diluted in 4% sheep serum (Sigma) for 30 minutes at
5 37°C. The ES colonies were incubated with the
6 primary antibodies (Oct4 from Santa Cruz
7 Biotechnologies, Heidelberg, Germany, final
8 concentration 10 µg/ml for 30 minutes at room
9 temperature. The ES colonies were washed twice
10 with PBS for 5 minutes and then incubated with the
11 secondary antibody (rat anti mouse immunoglobulin
12 (DAKO, Cambridgeshire, UK) used at 1:100 dilution)
13 for 30 minutes at room temperature. After that,
14 hES cells were washed again with PBS, incubated
15 with ABC/HRP solution for 25 minutes at room
16 temperature and washed again with PBS. The
17 detection was carried out by incubation with DAB
18 peroxidase (Enzo Life Sciences, NY) solution at
19 room temperature for 1 minute. Final washes were
20 done with distilled water. The bright field and
21 fluorescent images were obtained using a Zeiss
22 microscope and the AxioVision software (Carl Zeiss,
23 Jena, Germany).

24
25 **Comparison of hES cells-derived fibroblasts with**
26 **human foreskin fibroblasts.** To identify the nature
27 of feeder cells, hESdF were compared with human
28 foreskin fibroblasts (HFF; ATCC, Teddington, UK)
29 using flow-cytometry analysis. Briefly, hESdF were
30 harvested using 0.05% Trypsin/0.53M EDTA
31 (Invitrogen, Paisley, Scotland) and suspended in
32 staining buffer (PBS +5% FCS) at concentration 10^6

1 cells/ml. Hundred μ l of the cell suspension was
2 stained with 0.2 μ g of CD31 (PECAM-1), CD71
3 (Transferrin receptor), CD90 (Thy-1), and CD106
4 (VCAM-1) antibodies (all available from BD
5 Biosciences, Oxford, UK) at 4°C for 20 minutes.
6 Three washes in staining buffer were carried out
7 before staining with secondary antibody, goat anti-
8 mouse Ig-FITC (Sigma, Dorset, UK) used at 1:512
9 dilution at 4°C for 20 minutes. Cells were washed
10 again three times and resuspended in staining
11 buffer before being analysed with FACS Calibur (BD)
12 using the CellQuest software. 10,000 events were
13 acquired for each sample and propidium iodide
14 staining (1 μ g/ml) was used to distinguish live
15 from dead cells.

16
17 **Karyotype analysis of hES cells and hES cells-**
18 **derived fibroblasts.** The karyotype of hES cells
19 and hES cells-derived fibroblasts was determined by
20 standard G-banding procedure. A suitable protocol
21 is available at:
22 [http://www.slh.wisc.edu/cytogenetics/Protocols/Stai](http://www.slh.wisc.edu/cytogenetics/Protocols/Staining/G-Banding.html)
23 [ning/G-Banding.html](http://www.slh.wisc.edu/cytogenetics/Protocols/Staining/G-Banding.html)

24
25 **Reverse Transcription (RT)-PCR analysis.** The
26 reverse transcription was carried out using the
27 cells to cDNA II kit (Ambion, Huntingdon, UK)
28 according to manufacturer's instructions. In
29 brief, hES cells were submerged in 100 μ l of ice-
30 cold cell lysis buffer and lysed by incubation at
31 75°C for 10 minutes. Genomic DNA was degraded by
32 incubation with DNase I for 15 minutes at 37°C. RNA

1 was reverse transcribed using M-MLV reverse
2 transcriptase and random hexamers following
3 manufacturer's instructions. PCR reactions were
4 carried out using the following primers (Seq ID Nos
5 1 to 12):

6
7 OCT4 (F): 5' - GAAGGTATTCAGCCAAAC-3';
8 OCT4 (R): 5' - CTTAATCCAAAAACCCTGG-3';
9 REX1 (F): 5' - GCGTACGCAAATTAAAGTCCAGA-3';
10 REX1 (R): 5' - CAGCATCCTAAACAGCTCGCAGAAT-3';
11 NANOG (F): 5' - GATCGGGCCCGCCACCATGAGTGTGGATCCAGCTTG-3';
12 NANOG (R): 5' - GATCGAGCTCCATCTTCACACGTCTTCAGGTTG-3';
13 FOXD3F: 5' - GGAGGGAGGGGGCAATGCAC- 3';
14 FOXD3R: 5' - CCCCAGAGCTCGCCTACT -3'
15 TERT (F): 5' - CGGAAGAGTGTCTGGAGCAAGT-3':
16 TERT (R): 5' - GAACAGTGCCTTCACCCTCGA -3';
17 GAPDH (F): 5' - GTCAGTGGTGGACCTGACCT-3';
18 GAPDH (R): 5' - CACCACCCTGTTGCTGTAGC-3'.

19
20 Note that (F) and (R) refer to the direction of the
21 primers and designate forward and reverse direction
22 respectively.

23
24 PCR products were run on 2% agarose gels and
25 stained with ethidium bromide. Results were
26 assessed on the presence or absence of the
27 appropriate size PCR products. Reverse
28 transcriptase negative controls were included to
29 monitor genomic contamination.

30

31 **DNA Genotyping of hES cells and hES cells-derived**
32 **fibroblasts.** Total genomic DNA was extracted from

1 both hES cells and hES cells-derived fibroblasts.
2 DNA from both samples was amplified with 11
3 microsatellite markers: D3S1358, vWA, D16S539,
4 D2S1338, Amelogenin, D8S1179, D21S11, D18S51,
5 D19S433, TH01, and FGA (Chen Y et al., 2003, Cell
6 Res. 2003 Aug;13(4):251-63. full paper available at
7 [http://www.cell-research.com/20034/2003-116/2003-4-](http://www.cell-research.com/20034/2003-116/2003-4-05-ShengHZ.htm)
8 05-ShengHZ.htm) and analysed on an ABI 377 sequence
9 detector using Genotype software (Applied
10 Biosystems, Foster City, CA).

11

12 **Growth of hES cells on hESdF.** HES-NCL1 cells were
13 grown on γ -irradiated hESdF monolayer (75.000
14 cells/cm²) in ES medium containing Knockout-DMEM
15 (Invitrogen), 100 μ M β -mercaptoethanol (Sigma), 1
16 mM L-glutamine (Invitrogen), 100 mM non-essential
17 amino acids, 10% serum replacement (SR,
18 Invitrogen), 1% penicillin-streptomycin
19 (Invitrogen) and 4 ng/ml bFGF (Invitrogen). ES
20 medium was changed daily. HES cells were passaged
21 every 4-5 days by incubation in 1 mg/ml collagenase
22 IV (Invitrogen) for 5-8 minutes at 37°C or
23 mechanically dissociated and then removed to plates
24 with freshly prepared hESdF.

25

26 **Recovery of hESdF-conditioned medium.** Mitotically
27 inactivated HESdF were cultured in T-25 flask with
28 addition of ES medium for 10 days. hESdF-
29 conditioned medium was collected every day and then
30 frozen at -80°C.

31

1 **Growth of hES cells in feeder-free system using**
2 **hESdF-conditioned medium.** hES cells were passaged
3 and then removed to plates precoated with Matrigel
4 (BD, Bedford, MA) as previously described. ¹⁶ ES
5 media conditioned by hESdF was changed every 48
6 hours.

7
8 **Cryopreservation of hES cells and hESdF.** To see
9 whether frozen-thawed hESdF still support
10 undifferentiated growth of cryopreserved hES cells,
11 hESdF were frozen at -80°C using FCS supplemented
12 with 10% (v/v) dimethyl sulfoxide (Sigma). Clumps
13 of hES cells were frozen or vitrified using
14 protocol as previously described (see Reubinoff et
15 al., 2001, Hum Reprod 10:2187-2194). Mitotic
16 inactivation by using mitomycin C could
17 alternatively be used.

18
19 **Tumor formation in severe combined immunodeficient**
20 **(SCID) mice (Stefan).** Ten to fifteen clumps with
21 approximately 3000 hES cells in total were injected
22 in kidney capsule, subcutaneously in flank or in
23 the testis. After 21-90 days, mice were
24 sacrificed, tissues were dissected, fixed in Bouins
25 overnight, processed and sectioned according to
26 standard procedures and counterstained with either
27 haematoxylin and eosin or Weigerts stain. Sections
28 were examined using bright field light microscopy
29 and photographed as appropriate.

30

1 All procedures involving mice were carried out in
2 accordance with institution guidelines and
3 institution permission.
4

5 **Statistical analysis.** Cell numbers of Day 6 and Day
6 8 ICMs were compared using Wilcoxon rank-sum test.
7 The data are presented as mean \pm standard
8 deviation.
9

10 ***In vitro* differentiation of hES cells.** Colonies of
11 hES-NCL1 passage 21 were grown in feeder-free
12 conditions in ES medium. After 5 to 14 days
13 spontaneous differentiation was observed and
14 differentiated cells were passaged and cultured
15 under same conditions. Cells were fixed in 4%
16 paraformaldehyde in PBS (Sigma) for 30 minutes and
17 then permeabilised for additional 10 minutes with
18 0.1% Triton X (Sigma). The blocking step was 30
19 minutes with 2% FCS in PBS. Cells were incubated
20 with antibody against nestin (1:200; Chemicon) or
21 human alpha smooth muscle actin (1:50; Abcam,
22 Cambridge, UK) for additional 2 hours. Each
23 antibody was detected using corresponding secondary
24 antibodies conjugated to FITC. The nuclei of cells
25 were stained using propidium iodide for 5 minutes.
26

27 **Results**

28 Traditionally early blastocysts (Day 6) have been
29 used for the derivation of human ES cell line. We
30 developed a three - step culture system (see
31 Materials and Methods) which supports successfully
32 the development of late (Day 8) blastocysts.

1 Analysis of cell numbers of ICMs revealed that Day
2 8 blastocysts possess significantly ($P < 0.01$) more
3 ICM cells than Day 6 blastocysts (51.3 ± 9.6 vs.
4 36.8 ± 11.9 , respectively). In view of this result
5 we used day 8 blastocysts to derive human ES cell
6 lines. Of the 11 Day 2 donated embryos, 7 (63.6%)
7 blastocysts developed to Day 6. All 7 of these
8 blastocysts expanded or hatched on Day 8 after
9 transfer to G-BRLC medium. After isolation of ICMs
10 by immunosurgery, 3 primary hES cell colonies
11 showed visible outgrowth and one stable hES cell
12 line (ICL-NCL1) was successfully derived (Figs. 1C-
13 E).

14

15 When the hES cells were cultured in the absence of
16 feeder cells they spontaneously differentiated into
17 fibroblast-like cells, ie. long, flat cells with
18 elongated, condensed nucleus. We confirmed that
19 the differentiated cells were fibroblasts by
20 staining with a specific antibody to fibroblast
21 surface protein (AFSP) (Fig. 2C and D). Karyotyping
22 of the hES cells and hES cells-derived fibroblasts
23 revealed that both samples are normal female ($46 +$
24 XX , Figs. 2E and F). Microsatellite analysis
25 revealed that the hES cells and hES cells-derived
26 fibroblasts are indistinguishable from each other
27 and should be considered as autogenic (see Fig. 2G,
28 2H). We now have several batches of fresh and
29 frozen/thawed serially expanded hES cells-derived
30 fibroblasts which support hES cell culture even
31 after the twelfth passage but they are optimal
32 between second and eighth passages. Flow-cytometry

1 (Fig. 7) revealed that very few cells showed
2 expression of mesenchymal cell specific markers
3 CD106 (V-CAM1) and CD71 (transferring receptor) and
4 none expressed the endothelial specific cell marker
5 CD31 (PECAM-1). On the contrary, 94% and 82% of the
6 hESdF cells were stained with the CD44 and CD90
7 (THY-1) antibodies, respectively. Both antibodies
8 were also presented in human foreskin fibroblasts
9 (HFF; Fig. 7).

10
11 The hES-NCL1 line has been cultured on hES cell
12 derived fibroblasts (hESdF) for over 35 passages
13 and on Matrigel with hESdF conditioned medium for
14 13 passages. We found that hES cell colonies grown
15 on hES cell derived fibroblasts were dense, compact
16 and suitable for mechanical passaging with typical
17 morphology of hES cells (Fig. 4). Characterisation
18 studies demonstrated that hES cells cultured on hES
19 cells-derived fibroblasts or Matrigel with addition
20 of hESdF-conditioned medium expressed specific
21 surface markers: GTCM2, TRA1-60 and SSEA4, and
22 (Fig. 4A-H) and were positive for the expression of
23 *OCT-4*, *NANOG*, *FOXD3*, *REX-1* and *TERT* by RT-PCR (Fig.
24 5A). Expression of TG343 was also found in hES
25 cells grown on mouse feeder cells, and whilst not
26 tested in the hESdf grown cells would be expected
27 to be present. The fibroblast-like cells also
28 expressed the telomerase reverse transcriptase
29 (*TERT*) and *REX1* in early passages but none of the
30 other ES cell specific markers. Human ES cells
31 grafted into SCID mice consistently developed into
32 teratomas demonstrating the pluripotency of hES-

1 NCL1 cells grown on hESdF. Teratomas were primarily
2 restricted to the site of injection and their
3 histological examination revealed advanced
4 differentiation of structures representative of all
5 three embryonic germ layers, including cartilage,
6 skin, muscle, primitive neuroectoderm, neural
7 ganglia, secretory epithelia and connective tissues
8 (Fig. 6). When hES-NCL1 cells were cultured in
9 absence of feeders and Matrigel, spontaneous
10 differentiation into neuronal (Fig. 8A) and smooth
11 muscle (Fig. 8B) cells was observed.

1 / 8

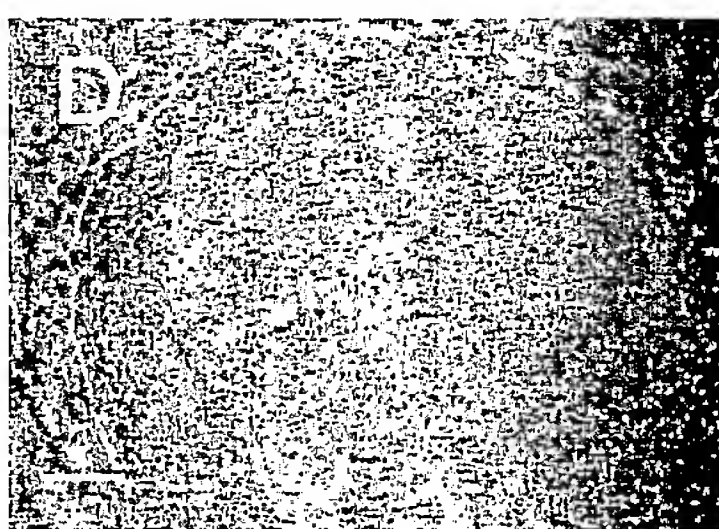
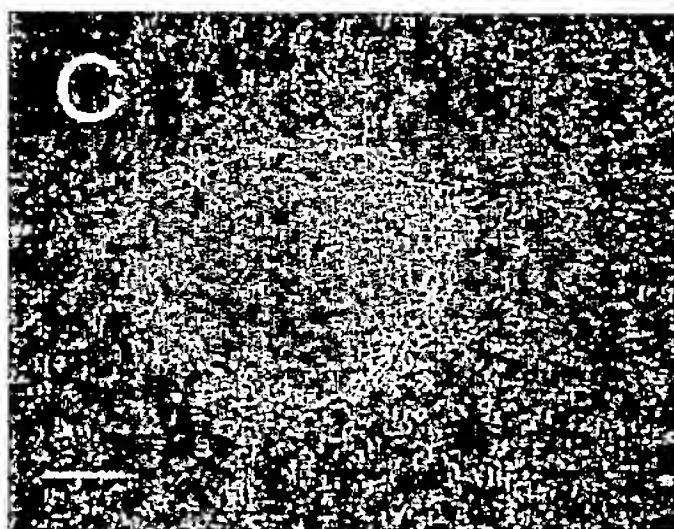
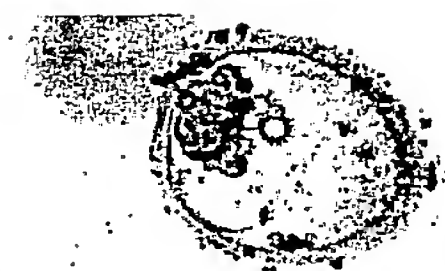
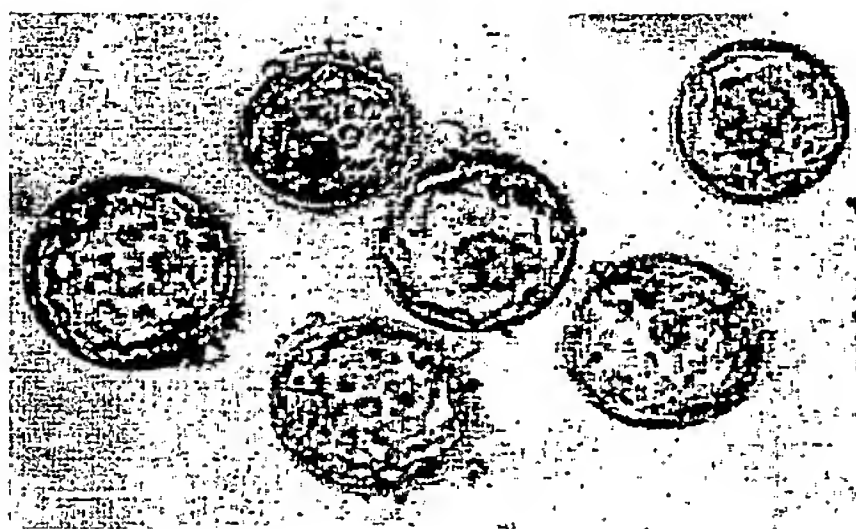


Fig. 1



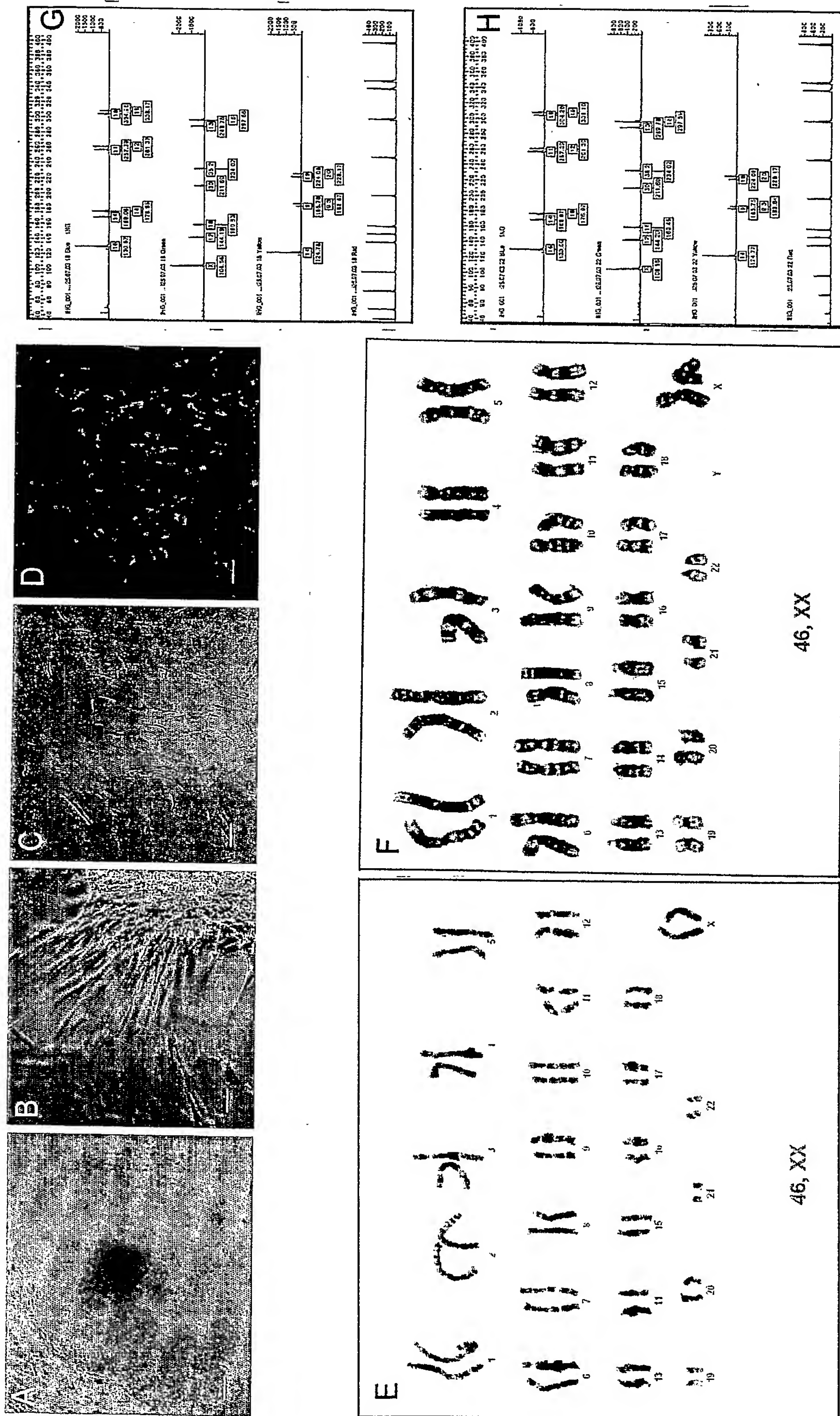


Fig. 2

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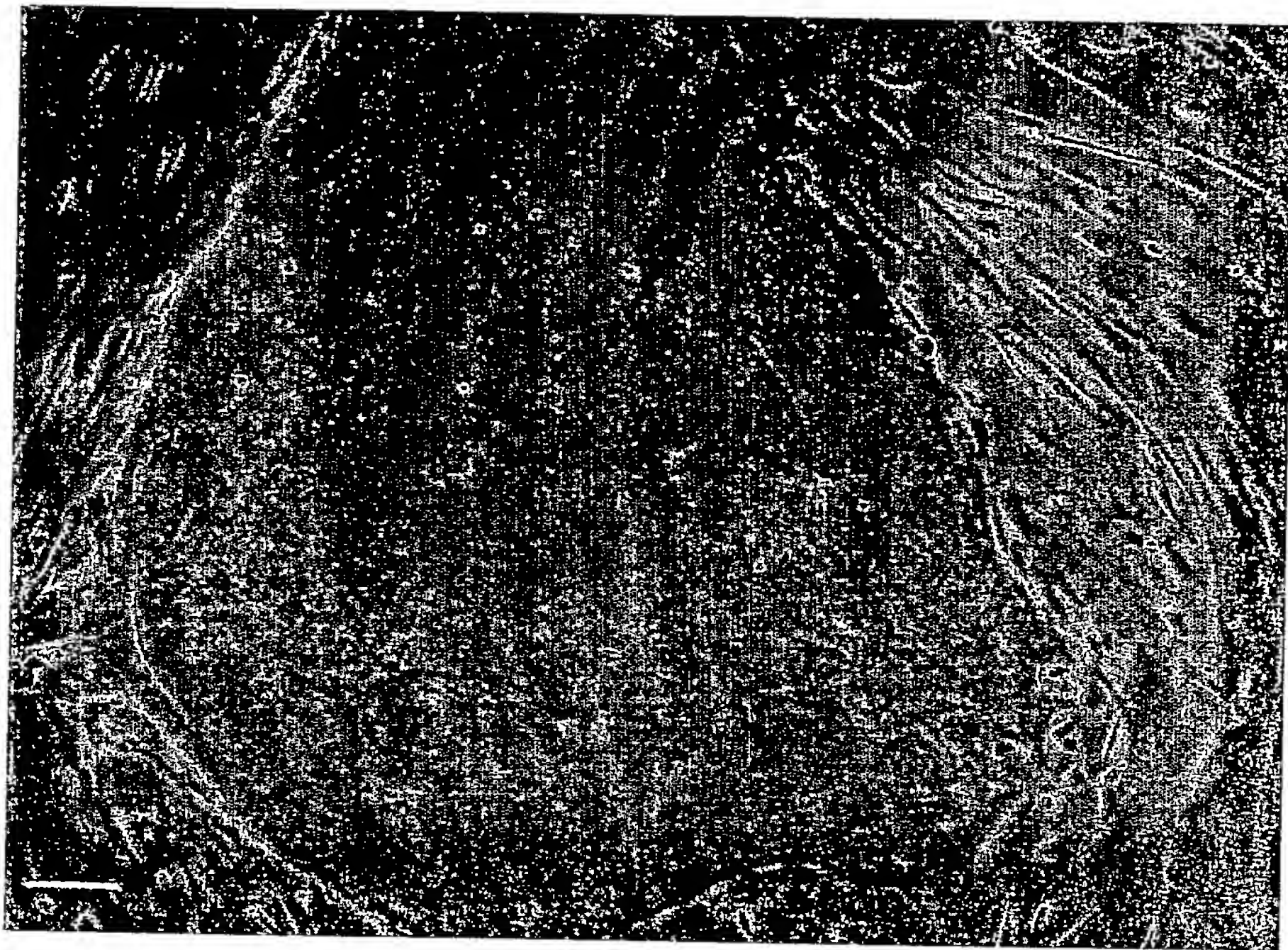


Fig. 3

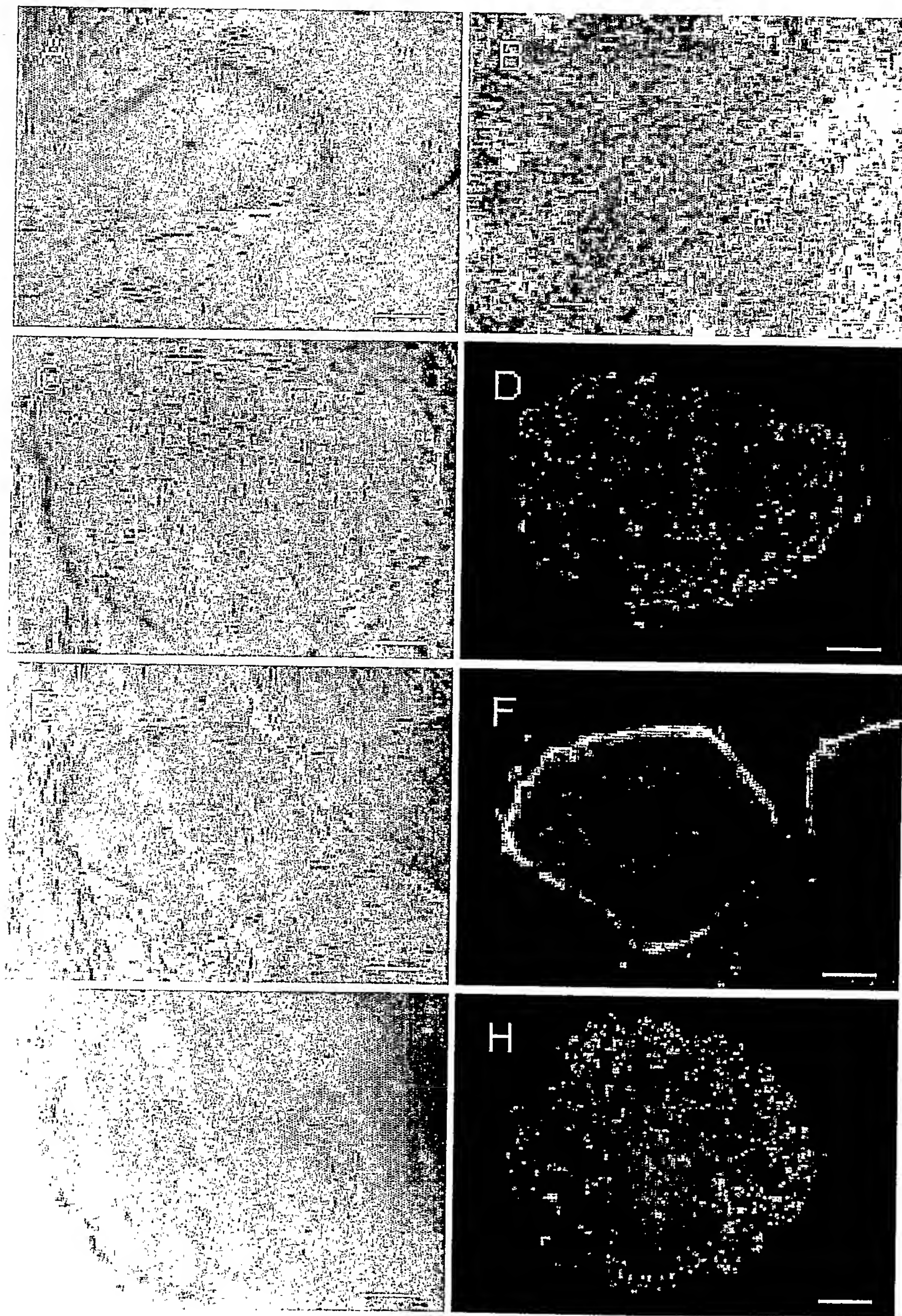
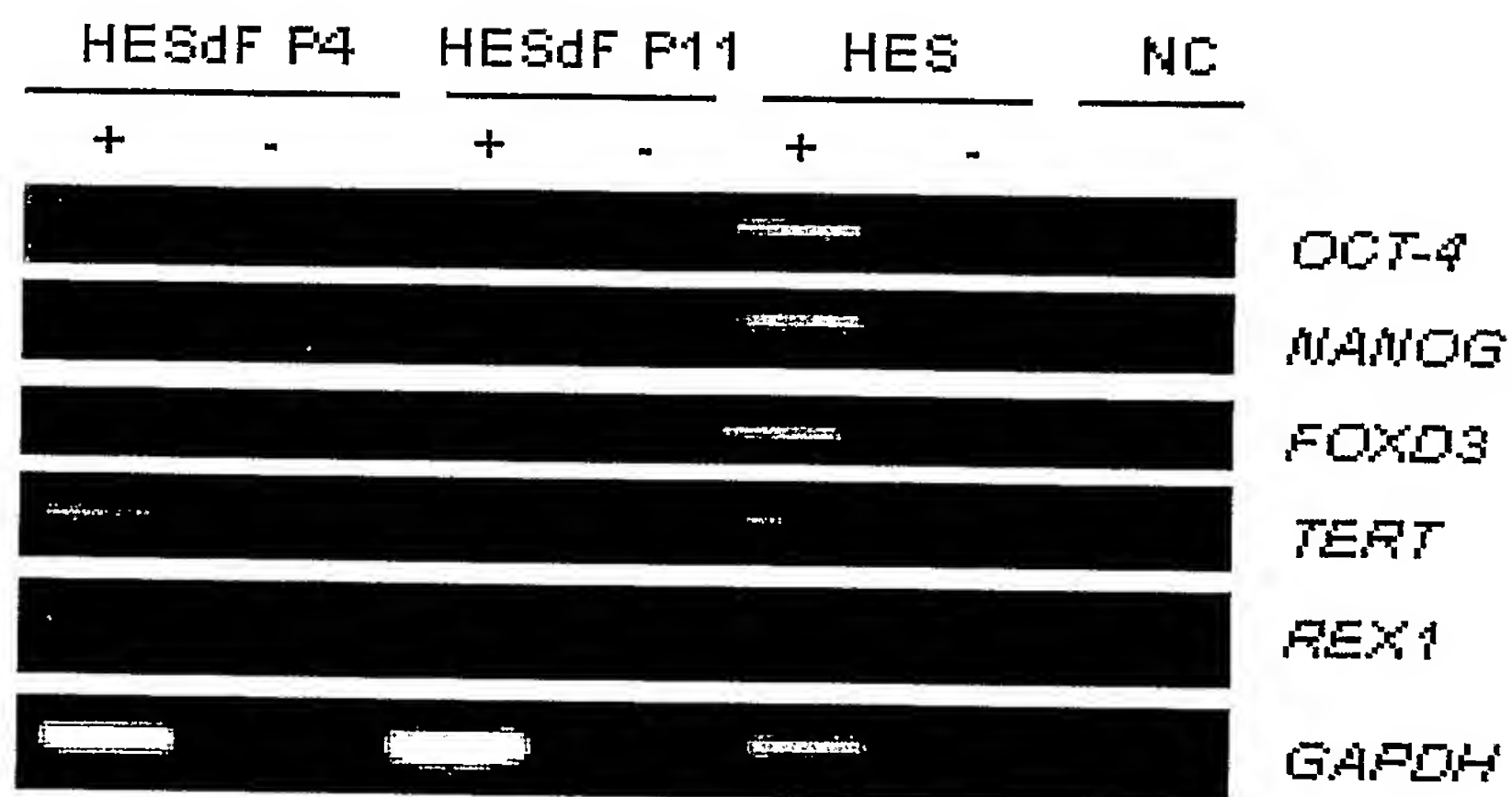


Fig. 4

A



B

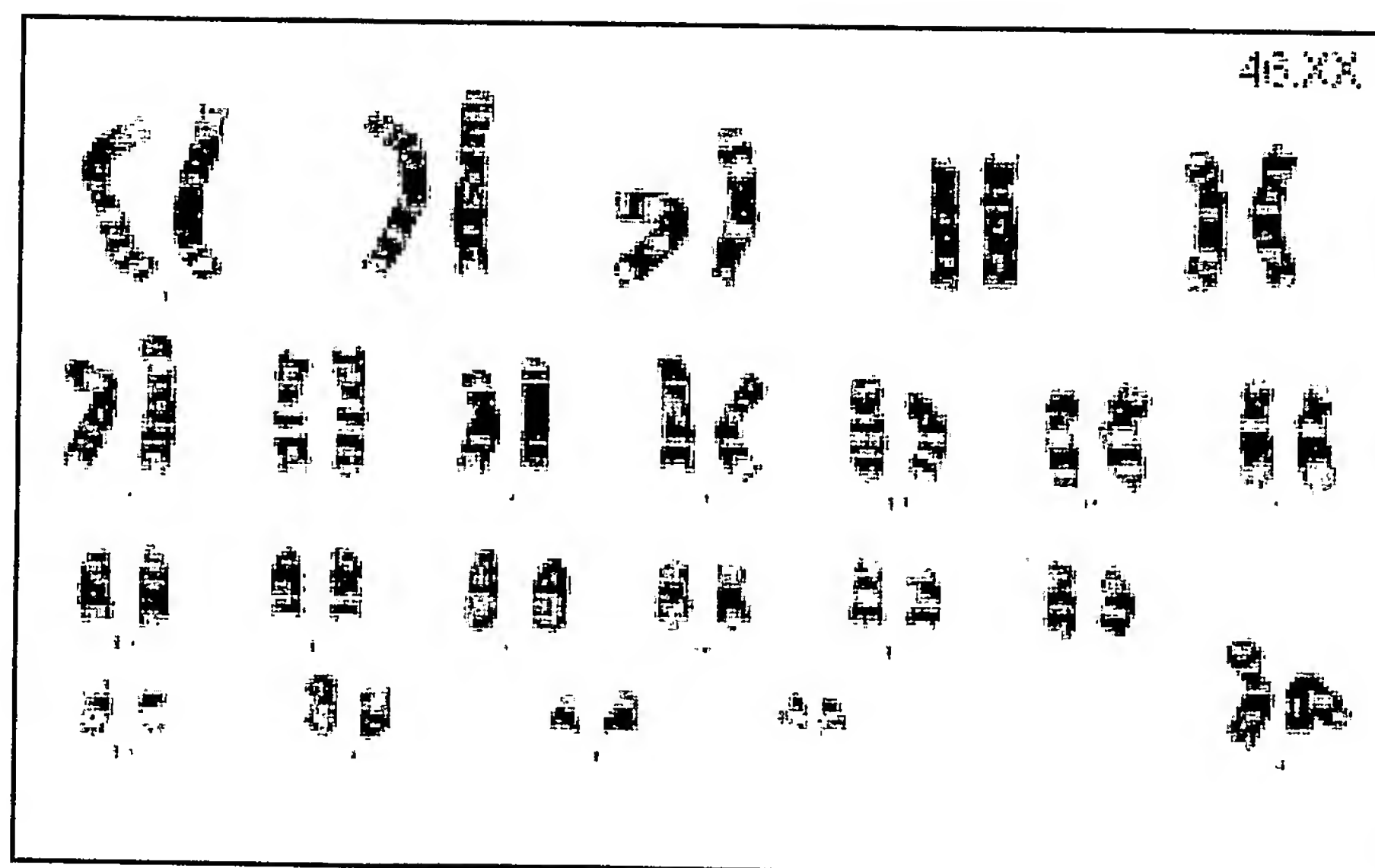


Fig. 5

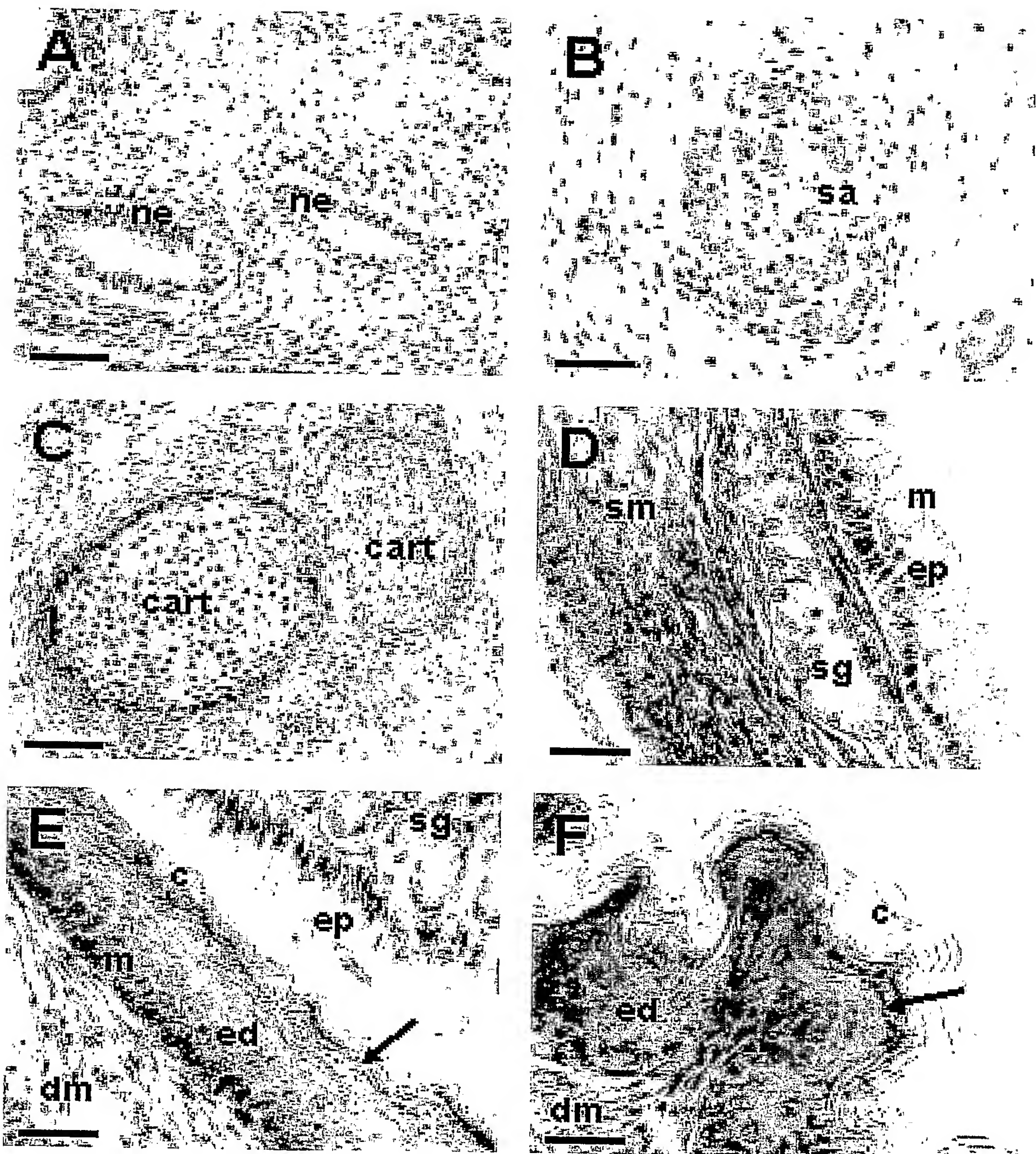


Fig. 6

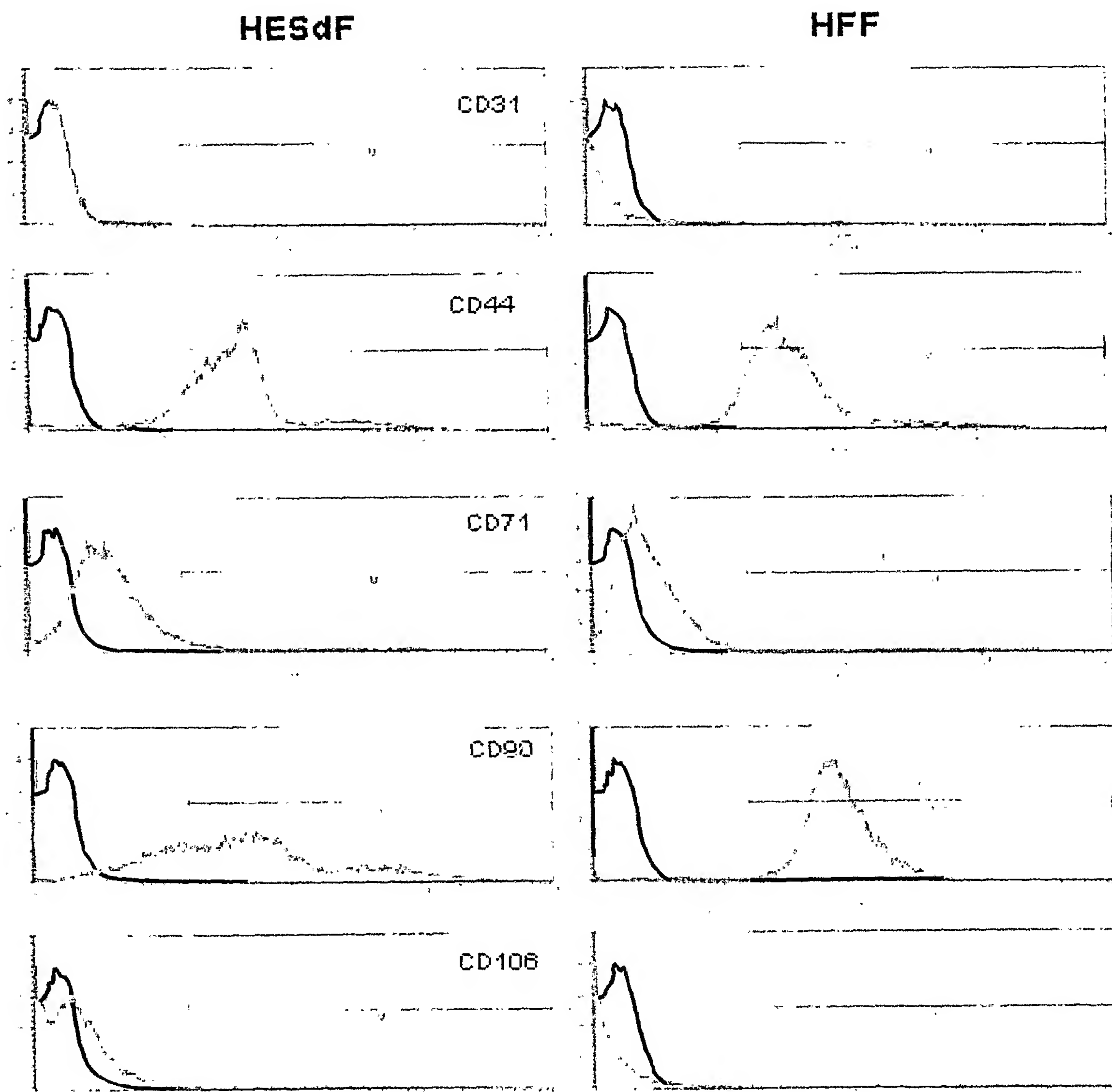


Fig. 7



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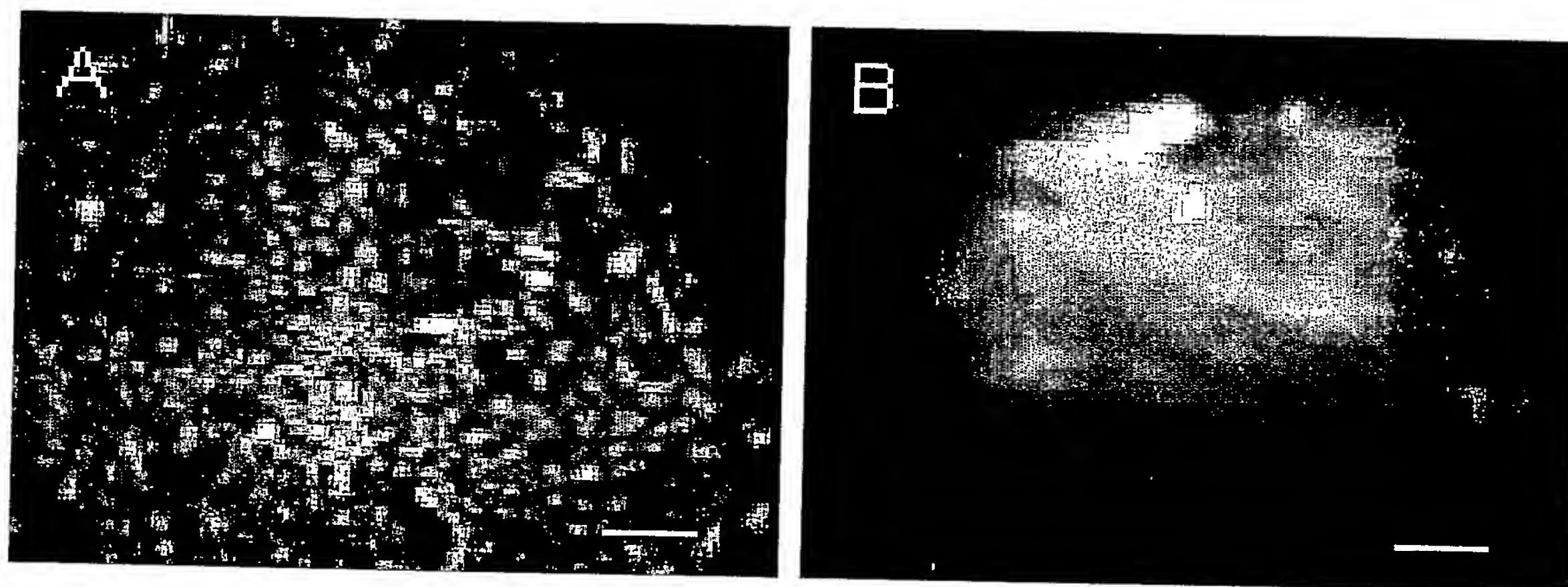


Fig. 8

